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The S7 gene and VP7 protein are highly conserved among temporally and geographically distinct American isolates of epizootic hemorrhagic disease virus

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Abstract

Complete sequences of genome segment 7 (S7) from six isolates of epizootic hemorrhagic disease virus serotype 1 (EHDV-1) and 37 isolates of serotype 2 (EHDV-2) were determined. These isolates were made between 1978 and 2001 from the southeast, mid-Atlantic, Midwest and intermountain United States. Analysis of the S7 sequence similarities showed 98.1% identity among the EHDV-1 isolates and 91.0% identity among the EHDV-2 isolates. Comparison of the deduced amino acid similarities showed an even greater degree of similarity among the isolates (100% among the EHDV-1 isolates and 98.9% identity among the EHDV-2 isolates). There was only 75.8% identity between the EHDV-1 and EHDV-2 isolates at the nucleic acid level; however, there was 93.7% identity between the two groups at the amino acid level. The ratio of non-synonymous to synonymous nucleotide indicates a strong selection for silent substitutions. There was no evidence for reassortment between EHDV-1 and EHDV-2 isolates. The high degree of conservation of S7 gene codons and the VP7 protein, suggests that little variation is allowed in preserving the function of this protein. The high degree of conservation also validates the use of diagnostic tests for EHDV based on S7 and VP7.

Keywords: EHDV; Sequence analysis; Phylogenetic relationships

Epizootic Hemorrhagic disease virus (EHDV) is in the genus *Orbivirus* in the family Reoviridae. Worldwide, there are at least eight distinct serotypes of EHDV (Gorman, 1992). Two serotypes, designated EHDV-1 (New Jersey strain) and EHDV-2 (Alberta strain), have been isolated in North America (Barber and Jochim, 1975). These viruses are structurally, antigenically and genetically related to the bluetongue virus (BTV) serogroup of orbiviruses. They cause an often fatal hemorrhagic disease of wild North American ungulates including white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*) and pronghorn antelope (*Antilocapra americana*) (Shope et al., 1960; Couvillion et al., 1981; Howerth et al., 2001), and there is evidence they may cause bluetongue-like illness in cattle (Omori et al., 1969; Campbell et al., 1978; Metcalf and Luedke, 1980). The mode of transmission for EHDV is similar to BTV and is mediated by biting midges in the genus *Culicoides* (Foster et al., 1977; Jones et al., 1977).

Genome segment 7 (S7) codes for the structural protein VP7 (Mecham and Dean, 1988). This protein is highly conserved between members of the BTV serogroup and serves as the basis for both antibody and polymerase chain reaction (PCR) based diagnostic procedures (Mecham and Wilson, 1994; Wilson et al., 2000). PCR tests based on S7 and competitive ELISA based on VP7 have been developed for the diagnosis of EHDV infections (Thevasagayam et al., 1996; Afshar et al., 1997; Mecham and Jochim, 2000). The reliability of these diagnostic procedures is influenced by the degree of genotypic and phenotypic variation among members of the sero-group. The genetic and phenotypic variation

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of S7 and VP7 among EHDV isolates is unknown. This report describes the sequence analysis of EHDV isolates from the southeast, mid-Atlantic, Midwest and intermountain United States obtained between 1978 and 2001. Phylogenetic analysis revealed some temporal grouping, but no obvious geographical grouping of virus isolates. There was a remarkably high degree of conservation of deduced VP7 amino acid sequences among the geographically and temporally distinct isolates.

Viruses were obtained from the Southeastern Cooperative Wildlife Disease Study, Athens, GA; the Wyoming State Veterinary Laboratory, Laramie, WY; the Colorado State University-Veterinary Diagnostic Laboratory, Fort Collins, CO; and the Arthropod-borne Animal Diseases Research Laboratory (ABADRL). The state, host and year of isolation of the viruses analyzed in this study are shown in Tables 1 and 2. All virus isolates were passed in cell culture one to three times. Published sequences for the prototype North American strains of EHDV-1 and EHDV-2 were included for comparison (Iwata et al., 1992; Wilson et al., 2000).

The viral dsRNA was purified from infected BHK-21 cell cultures using the Qiagen RNA/DNA purification system (Qiagen Inc.). The cDNA template was prepared by reverse transcription and PCR with terminal primers based on the published sequences of S7 for the prototype strains of EHDV-1 and EHDV-2 (Iwata et al., 1992; Wilson et al., 2000). The PCR products were sequenced directly using an ABI 3100 automated sequencer (Applied Biosystems Inc.; Smith et al., 1986).

Sequence data were compiled with SEQMAN software (DNA Star Inc., Madison, WI) and analyzed with VECTOR NTI version 8 software (InforMax Inc., Fredrick, MD). Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 (Kumar et al., 2001) and Phylogenetic Analysis Using Parsimony (PAUP), version 4.0b10 (Swofford, 1997). The ratio of non-synonymous to synonymous nucleic acid substitutions (dN/dS) was done with MEGA using the Nei-Gojobori method based on p-distance.

The S7 genome segment of all EHDV isolates was 1162 base pairs in length and encoded a deduced protein of 349 amino acids. There was 98.1% identity at the nucleic acid level and 100% identity at the amino acid level among the six EHDV-1 isolates. Comparison of these sequences to the published sequence of S7 for EHDV-1 showed 96.1 and 98.9% identity at the nucleic acid and amino acid levels, respectively. The ratio of non-synonymous to synonymous nucleic acid substitutions was 0.0329. The non-synonymous substitutions resulted in four amino acid differences in the deduced VP7 sequence of the prototype EHDV-1 isolate compared with the VP7 sequence of our six EHDV-1 isolates. These changes were located at position 37 (Val/Ile), position 149 (Val/Ile), position 238 (Leu/Gln), and position 315 (Ala/Ser).

Table 1
List of EHDV-2 isolates and Gen Bank accession numbers for S7 sequences

Isolate designation	State	Host	Year	Accession number
EHDV 2 NA[a]			1973	AF188643[a]
cc86 78	GA	WTD	1978	AY261492
cc96 80	GA	WTD	1980	AY261484
cc87 90	GA	WTD	1990	AY261477
cc211 91	WY	WTD	1991	AY261473
cc118 93	WVA	WTD	1993	AY261475
cc131 93	WVA	WTD	1993	AY261474
cc110 93	AL	WTD	1993	AY261478
cc140 93	SC	WTD	1993	AY261476
Gen 3	AR	WTD	1996	AY261485
cc148 96	AL	WTD	1996	AY261487
cc152 96	VA	WTD	1996	AY261494
cc139 96	MO	WTD	1996	AY261505
cc129 96b	IN	WTD	1996	AY261489
W7606	WY	WTD	1996	AY261472
W8288	WY	WTD	1996	AY261471
W8289	WY	WTD	1996	AY261470
W8578	WY	WTD	1996	AY261469
cc172 98	TN	WTD	1998	AY261479
cc193 98	TN	WTD	1998	AY261480
Cow 17	MO	Cow	1998	AY261488
cc184 98	KS	WTD	1998	AY261490
cc155 99	MS	WTD	1999	AY261501
cc134 99	MD	WTD	1999	AY261491
MOC 1	IA	Cow	1999	AY261486
MOC 2	IA	Cow	1999	AY261504
587 3	NC	WTD	2000	AY261481
cc142 00	SC	WTD	2000	AY261493
cc127 00	GA	WTD	2000	AY261495
cc135 00	TX	WTD	2000	AY261482
cc149 00	VA	WTD	2000	AY261502
cc159 00	MD	WTD	2000	AY261503
cc137 00	KS	WTD	2000	AY261483
012 11040	CO	Elk	2001	AY261500
012 09802	CO	Elk	2001	AY261496
01W8009	WY	WTD	2001	AY261497
01W8425	WY	WTD	2001	AY261498
01W8477	WY	WTD	2001	AY261499

[a] Wilson et al., 2000.

Table 2
List of EHDV-1 isolates and Gen Bank accession numbers for S7 sequences

Isolate designation	State	Host	Year	Accession number
EHDV 1 NA[a]				D10766[a]
cc128 99	GA	WTD	1999	AY261507
Forest	GA	WTD	1999	AY261510
Cc133 99	NC	WTD	1999	AY261508
Cc136 99	VA	WTD	1999	AY261509
cc124 99	MD	WTD	1999	AY261506
Kerr 72	TX	WTD	2000	AY261511

[a] Iwata et al., 1992.

The 37 EHDV-2 isolates were 91% identical at the nucleic acid level and 98.9% identical at the deduced amino acid level. This did not change when these sequences were compared with the published sequence for S7 of the EHDV-2 prototype strain. The non-identical nucleic acids and amino acids of the EHDV-2 isolates were scattered throughout S7 and the VP7 protein. Calculation of the non-synonymous to synonymous ratio gave a value of 0.0097. There were only four amino acid differences between all the EHDV-2 isolates; these were located at positions 56 (Glu/Asp), 152 (Ala/Ser), 249 (Tyr/Phe), and 319 (Ala/Thr). These changes were restricted to ten of the virus isolates; and each of these isolates had only one amino acid change from the consensus. All the amino acid changes were conservative or semi-conservative; and none resulted in a significant change in overall predicted charge or hydrophobic nature of VP7.

Comparison of the S7 sequences and the deduced VP7 amino acid sequences showed 75.8% nucleic acid identity and 93.1% amino acid identity between all the EHDV-1 and EHDV-2 isolates. The ratio of non-synonymous to synonymous nucleotide substitutions was 0.0316, and suggests that synonymous changes are approaching saturation.

Phylogenetic analysis of all the nucleic acid sequence data using MEGA and the Kimura 2-parameter model is shown in Fig. 1. Two main clades, representing EHDV-1 and EHDV-2 isolates, were generated. There was no evidence of reassortment between the two groups, since all the S7 sequences segregated into their respective clade. Phylogenetic analysis was focused on the EHDV-2 isolates to determine any temporal or geographical clustering based on S7 sequences (Fig. 2). There was some grouping of viruses based on year of isolation, which was not absolute; however, there was no obvious genetic grouping based on geographical location of virus isolation. Analysis using PAUP and maximum parsimony gave similar results (data not shown). Meaningful phylogenetic analysis of the EHDV-2 isolates based on the deduced amino acid sequences of VP7 was not possible, because of the small number of changes among the isolates. However, when the amino acid changes were mapped onto this phylogeny, the same amino acid changes occurred in virus isolates that grouped together (Fig. 2). Phylogenetic analysis on just the EHDV-1 isolates was not performed, since there were only six isolates made in 1999 and 2000 from a limited geographical area.

Sequence similarity and the low rate of non-synonymous nucleotide substitutions among the virus isolates imply that there is little allowed amino acid variation in preserving the function of VP7. Amino acid composition, amino acid order, charge and hydrophobicity may all play a role in maintaining the function of this protein. Structural studies indicate very specific interactions between VP7 and VP3 to form the BTV core particle (Hewat et al., 1992; Grimes et al., 1997). In addition to forming the core structure, VP7 interacts

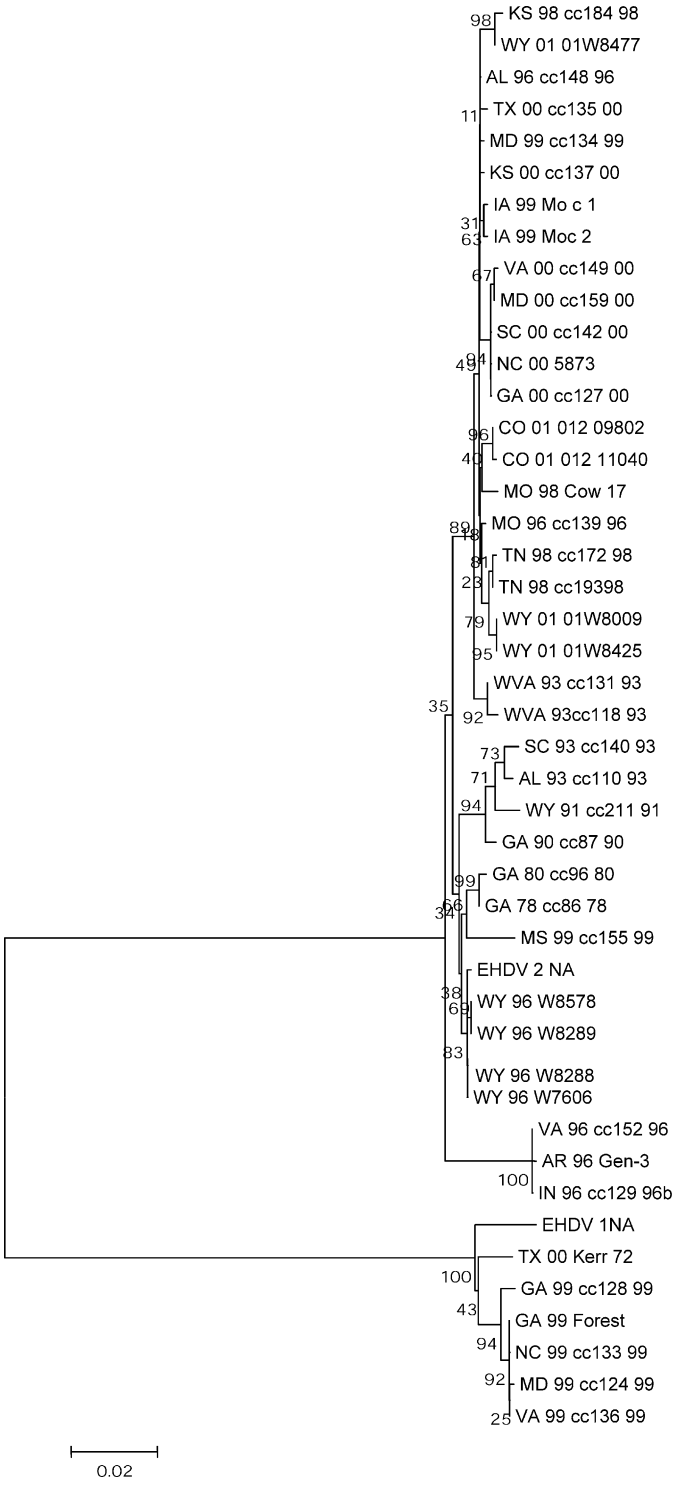

Fig. 1. Phylogenetic analysis of the EHDV-1 and EHDV-2, S7 sequences. The numbers indicate bootstrap confidence values after 500 replications. The tree was generated using MEGA and the following analysis parameters: gamma distance measure ($\alpha = 1$), Kimura 2-parameter model and neighbor-joining bootstrap analysis (Kumar et al., 2001).

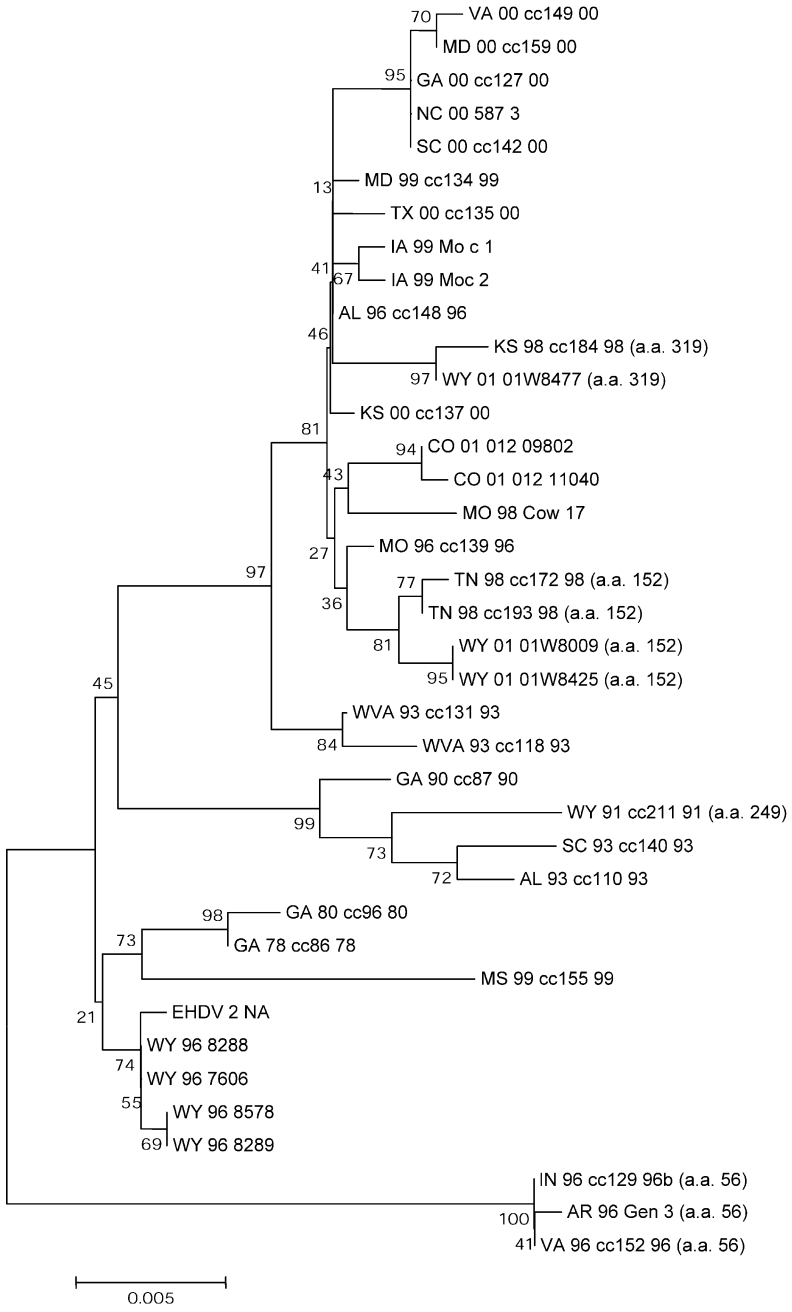

Fig. 2. Phylogenetic analysis of the EHDV-2, S7 sequences. The numbers indicate bootstrap confidence values after 500 replications. The tree was generated using MEGA and the following analysis parameters: gamma distance measure ($\alpha = 1$), Kimura 2-parameter model and neighbor-joining bootstrap analysis (Kumar et al., 2001). The position of amino acid changes from the consensus is indicated in parentheses opposite the respective virus isolate.

with VP5 and VP2 to form the outer capsid layer of BTV (Hewat et al., 1994). The infectivity of bluetongue virions modified by protease treatment and core particles in insect systems suggests that VP7, either alone or in combination with VP2 and VP5, plays a role in initiation of infection in *Culicoides* (Mertens et al., 1996). This is further supported by the demonstration that VP7 binds a *Culicoides* membrane protein (Xu et al., 1997; Tan et al., 2001).

EHDV has also been isolated from *Culicoides sonorensis*, which is the primary vector for BTV in the US (Foster et al., 1980; Tabachnick, 1996; Holbrook et al., 2000); however, it is not known if other species of *Culicoides* are competent for EHDV. In addition to *C. sonorensis*, *Culicoides variipennis* is present throughout the southeast, mid-Atlantic and Midwest. Genetic topotypes of BTV, based on S7 sequence analysis, did not correlate with the predominant species of *Culicoides*

present in particular geographical locations (Wilson et al., 2000). Thus, conservation of VP7 may be necessary to maintain its function in infection of the vector. This is supported by the high degree of amino acid conservation in VP7 of all the EHDV isolates in the present study. The significance of allowable changes in VP7 in terms of its function in infection of the insect and its role in virus structure and assembly remain to be determined.

A number of PCR and antigen configured diagnostic tests for EHDV are based on S7 and VP7. The highly conserved nature of S7 and VP7 among EHDV isolates validates their use in these diagnostic assays. Information on the complete S7 sequence of these virus isolates provides a data base that will allow the selection of primers of desired specificity for use in detection assays.

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References

Afshar, A., Anderson, J., Nielsen, K.H., Pearson, J.E., Trotter, H.C., Woolhouse, T.R., Thevasagayam, J.A., Gall, D.E., Lapointe, J.M., Gustafson, G.A., 1997. Evaluation of a competitive ELISA for detection of antibodies to epizootic hemorrhagic disease virus of deer. J. Vet. Diagn. Invest. 9, 309–311.

Barber, T.L., Jochim, M.M., 1975. Serotyping bluetongue and epizootic hemorrhagic disease virus strains. Proc. Am. Assoc. Vet. Lab. Diagn. 18, 149–162.

Campbell, C.H., Barber, T.L., Jochim, M.M., 1978. Antigenic relationship of ibaraki, bluetongue, and epizootic hemorrhagic disease viruses. Vet. Microbiol. 3, 15–22.

Couvillion, C.E., Nettles, V.F., Davidson, W.R., Pearson, J.E., Gustafson, G.A., 1981. Hemorrhagic disease among white-tailed deer in the southeast from 1971 through 1980. Proc. US Anim. Health Assoc. 85, 522–537.

Foster, N.M., Breckon, R.D., Luedke, A.J., Jones, R.H., 1977. Transmission of two strains of epizootic hemorrhagic disease virus in deer by *Culicoides variipennis*. J. Wildlife Dis. 13, 9–16.

Foster, N.M., Metcalf, H.E., Barber, T.L., Jones, R.H., Luedke, A.J., 1980. Bluetongue and epizootic hemorrhagic disease virus isolation from vertebrate and invertebrate hosts at a common geographic site. J. Am. Vet. Med. Assoc. 176, 126–129.

Gorman, B.M., 1992. An overview of the orbiviruses. In: Walton, T.E., Osburn, B.I. (Eds.), Bluetongue, African Horse Sickness and Related Orbiviruses. CRC Press, Boca Raton, FL, pp. 335–347.

Grimes, J.M., Jakana, J., Ghosh, M., Basak, A.K., Roy, P., Chiu, W., Stuart, D.I., Prasad, B.V., 1997. An atomic model of the outer layer of the bluetongue virus core derived from X-ray crystallography and electron cryomicroscopy. Structure 5, 885–893.

Hewat, E.A., Booth, T.F., Loudon, P.T., Roy, P., 1992. Three-dimensional reconstruction of baculovirus expressed bluetongue virus core-like particles by cryo-electron microscopy. Virology 189, 10–20.

Hewat, E.A., Booth, T.F., Roy, P., 1994. Structure of correctly self-assembled bluetongue virus-like particles. J. Struct. Biol. 112, 183–191.

Holbrook, F.R., Tabachnick, W.J., Schmidtman, E.T., McKinnon, C.N., Bobian, R.J., Grogan, W.L., 2000. Smypatry in the *Culicoides variipennis* complex (Diptera: ceratopogonidae): a taxonomic reassessment. J. Med. Entomol. 37, 65–67.

Howerth, E.W., Stallknecht, D.E., Kirkland, P.D., 2001. Bluetongue, epizootic hemorrhagic disease, and other Orbivirus-related diseases. In: Williams, E.S., Barker, I.K. (Eds.), Infectious Diseases of Wild Mammals. Iowa State Press, Ames, IA, pp. 77–97.

Iwata, H., Chuma, T., Roy, P., 1992. Characterization of the genes encoding two of the major capsid proteins of epizootic haemorrhagic disease virus indicates a close genetic relationship to bluetongue virus. J. Gen. Virol. 73, 915–924.

Jones, R.H., Roughton, R.D., Foster, N.M., Bando, B.M., 1977. Culicoides, the vector of epizootic hemorrhagic disease in white-tailed deer in Kentucky in 1971. J. Wildlife Dis. 13, 2–8.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. Molecular Evolutionary Genetics Analysis Software. Arizona State University, Tempe, AZ.

Mecham, J.O., Dean, V.C., 1988. Protein coding assignment for the genome of epizootic haemorrhagic disease virus. J. Gen. Virol. 69, 1255–1262.

Mecham, J.O., Jochim, M.M., 2000. Development of an enzyme-linked immunosorbent assay for the detection of antibody to epizootic hemorrhagic disease of deer virus. J. Vet. Diagn. Invest. 12, 142–145.

Mecham, J.O., Wilson, W.C., 1994. Strategies for improved bluetongue diagnostic strategies for improved bluetongue diagnostics. Proc. US Anim. Health Assoc. 98, 43–50.

Mertens, P.P., Burroughs, J.N., Walton, A., Wellby, M.P., Fu, H., O'Hara, R.S., Brookes, S.M., Mellor, P.S., 1996. Enhanced infectivity of modified bluetongue virus particles for two insect cell lines and for two Culicoides vector species. Virology 217, 582–593.

Metcalf, H.E., Luedke, A.J., 1980. Bluetongue and related viruses. Bov. Pract. 15, 188–191.

Omori, T., Inaba, Y., Morimoto, T., Tanaka, Y., Kono, M., 1969. Ibaraki virus, an agent of epizootic disease of cattle resembling bluetongue. II. Isolation of the virus in bovine cell culture. Jpn. J. Microbiol. 13, 159–168.

Shope, R.E., MacNamara, L.G., Mangold, R., 1960. A virus induced epizootic hemorrhagic disease of the Virginia white-tailed deer. J. Exp. Med. 111, 155–170.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughs, P., Dodd, C., Connell, C.R., Heines, C., Kent, S.B., Hood, L.E., 1986. Flourescence detection in automated DNA sequence analysis. Nature 321, 674–679.

Swofford, D.L., 1997. PAUP (Phylogenetic Analysis Using Parsimony). Smithsonian Institution.

Tabachnick, W.J., 1996. *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. Annu. Rev. Entomol. 41, 23–43.

Tan, B.H., Nason, E., Staeuber, N., Jiang, W., Monastryrskaya, K., Roy, P., 2001. RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to culicoides cells. J. Virol. 75, 3937–3947.

Thevasagayam, J.A., Woolhouse, T.R., Mertens, P.P., Burroughs, J.N., Anderson, J., 1996. Monoclonal antibody based competitive ELISA for the detection of antibodies against epizootic haemorrhagic disease of deer virus. J. Virol. Methods 57, 117–126.

Wilson, W.C., Ma, H.C., Venter, E.H., van Djik, A.A., Seal, B.S., Mecham, J.O., 2000. Phylogenetic relationships of bluetongue viruses based on gene S7. Virus Res. 67, 141–151.

Xu, G., Wilson, W., Mecham, J., Murphy, K., Zhou, E.M., Tabachnick, W., 1997. VP7: an attachment protein of bluetongue virus for cellular receptors in *Culicoides variipennis*. J. Gen. Virol. 78, 1617–1623.